2004

Evaluation of rough Brucella strains as vaccines for brucellosis and pseudorabies in swine

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EVALUATION OF ROUGH *BRUCELLA* STRAINS AS VACCINES FOR BRUCELLOSIS AND PSEUDORABIES IN SWINE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Pathobiological Sciences

by
Lorraine Harrow Molin
B.S., Louisiana State University, 2001
December 2004

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Philip Elzer for the opportunity to work under his direction for the past few years. His knowledge and guidance have been invaluable, and he has assisted me in obtaining the skills, discipline, and sense of humor required for success. I would also like to thank my committee members, Dr. Frederick Enright and Dr. Dennis French, for their guidance and support throughout this project.

I would like to thank Mrs. Sue Hagius and Mr. Joel Walker, who have provided immeasurable assistance in every aspect of this project.

I would like to thank Dr. Phillip Hoyt for bleeding the pigs and for his advice, support, and guidance. I would also like to thank Dr. Sara Lyle for ultrasounding the gilts.

I would like to thank the past and present members of the Elzer Laboratory, including Quinesha Perry, Ryan Hebert, Camille Walker, Lindsey Ding, Holly Landes, Natalie Cooper, Chad Towns, and Dr. Christopher Roberts, for their assistance with the animals and within the laboratory.

I would also like to thank Dr. Ramesh Vemulapalli of Purdue University for providing the plasmid and Dr. Gerhardt Schurig for his assistance and advice.

Finally, I would like to thank my husband, Brett, for his undying support and love, without which this project would not have been possible.

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ABSTRACT

Brucellosis and pseudorabies lead to abortion in pregnant sows and are perpetuated by feral swine reservoirs. A multivalent oral vaccine for these diseases would improve vaccination and eradication programs worldwide. Previous studies have shown that the rough attenuated *Brucella* strains RB51 and VTRS1, when administered subcutaneously to swine, stimulate host immune responses, transiently colonize tissues, and provide partial protection against virulent *B. suis* infection in pregnant sows. A plasmid encoding for the pseudorabies virus glycoprotein D (PRV gD) has also been added to these strains as part of this project. This study evaluates the use of these strains as oral vaccines for swine brucellosis and investigates the ability of these vaccines to colonize lymph nodes and stimulate the production of antibodies against rough *Brucella* antigens and PRV gD. Orally administered VTRS1 stimulated a higher immune response than RB51 in a shorter period of time, transiently colonized lymph nodes, and did not lead to the production of anti-O-polysaccharide (OPS) antibodies that interfere with brucellosis diagnostic tests. Both RB51 and VTRS1 provided substantial litter protection in orally vaccinated sows challenged with virulent *B. suis*; however, VTRS1, unlike RB51, also provided partial protection in the sow. These studies support the use of orally administered VTRS1 as an efficacious vaccine for swine brucellosis. The addition of a plasmid encoding for PRV gD to these strains created the multivalent vaccines RB51+gD and VTRS1+gD. Compared to the above strains, the addition of the plasmid did not alter immune response stimulation to rough *Brucella* antigens and these vaccines were safe when administered to pregnant sows. Swine vaccinated with RB51+gD or VTRS1+gD exhibited low immune responses to the gD antigen and delayed tissue colonization by the vaccine strains. It was found that plasmid addition slowed the growth of the vaccine strains in the laboratory, which may account for the suppressed tissue

colonization and immune responses to the PRV gD antigen in swine. Further studies are necessary to evaluate the use of these strains as multivalent vaccines for brucellosis and pseudorabies in swine.

CHAPTER 1: INTRODUCTION

Following an overview of the research problem and a review of the literature, the generation of the multivalent vaccine strains VTRS1+gD and RB51+gD via the addition of a plasmid encoding for the pseudorabies virus glycoprotein D to brucellosis vaccine strains *B. abortus* strain RB51 and *B. suis* strain VTRS1 will be described. Serological responses to orally administered VTRS1 and RB51 and the colonization of orally and subcutaneously administered VTRS1 in sexually immature swine will be characterized and compared to previously collected data. The transformed strains VTRS1+gD and RB51+gD will also be discussed in terms of their stimulation of the immune response and tissue colonization and pathogenicity in pregnant sows when administered orally or subcutaneously to swine. Finally, the protection of VTRS1 and RB51 against virulent *B. suis* challenge in sows will be evaluated.

Overview of the Research Problem

Brucella suis and pseudorabies virus (PRV) are perpetuated by feral swine reservoirs that readily transmit these diseases to domestic herds (Hahn et al 1997, Dees 1999). The presence of over two million feral swine in the United States alone complicates vaccination and eradication programs, including routine surveillance and culling of infected animals (Conger 1999). Therefore, an orally administrable, multivalent vaccine for both *B. suis* and PRV would be ideal for this target population.

Brucella suis infection in swine can result in abortion and both male and female infertility (Deyoe and Manthei 1975). There is currently no commercially available vaccine for swine brucellosis; however, the attenuated strains RB51 and VTRS1 have been shown by previous studies to provide protection against virulent *B. suis* infection when administered subcutaneously and do not interfere with current diagnostic tests (Hagius et al 1995, Hagius et al 2001).

Pseudorabies virus, like *B. suis*, may also cause abortion, stillborn, macerated, or weak piglets, and while a commercial vaccine is available it is often not utilized due to difficulty in distinguishing vaccinates from infected animals during routine screening (Gustafson 1981). The use of PRV glycoprotein D (gD) as a potential subunit vaccine has been shown to protect mice against virulent PRV challenge when administered via a variety of vectors (Ishii et al 1988, Marchioli et al 1987, Shiau et al 2001), and vaccination of swine with PRV gD DNA provided partial protection against virulent challenge (Haagmans et al 1999).

The use of gD administered via VTRS1 or RB51 would ideally provide protection against both pseudorabies and swine brucellosis. Oral administration of these multivalent vaccines would allow for more widespread vaccination of the feral swine population without interference with sero-diagnostic tests.

Research Objectives

This report describes the work performed in order to accomplish the following research objectives:

1. Evaluation of two potential vaccine strains for *B. suis*, RB51 and VTRS1, in terms of tissue colonization, generation of immune responses, and protection against virulent *B. suis* challenge when administered orally to swine.
2. Transformation of RB51 and VTRS1 with a plasmid encoding for the PRV gD, creating potential multivalent vaccine strains RB51+gD and VTRS1+gD. Evaluation of these transformed strains in terms of tissue colonization, fetal pathogenesis, and generation of immune responses in swine when administered either subcutaneously or orally.

CHAPTER 2: REVIEW OF THE LITERATURE

Brucella Species

The genus *Brucella* consists of Gram-negative, facultative intracellular bacteria that reside primarily within macrophages. These bacteria are non-acid-fast, non-motile, non-spore-forming, short aerobic rods which often require increased carbon dioxide tension for growth, with colonies rarely appearing within the first forty-eight hours of incubation. There are currently six defined species that manifest themselves in a variety of hosts and are characterized based on dye and host susceptibilities. *Brucella abortus* is primarily responsible for infecting cattle and North American wild ruminants while *B. melitensis* infects goats and sheep, *B. ovis* infects sheep, *B. suis* infects swine and reindeer, *B. canis* infects dogs, and *B. neotomae* infects the desert wood rat (Timony et al 1988).

Of the six defined *Brucella* species only four are zoonotic in nature: *B. melitensis, B. suis, B. abortus,* and *B. canis*. Human infection results in undulant fever, a flu-like illness with symptoms of weakness, night sweats, and a cycling fever (Timony et al 1988). Due to the ability of these agents to illicit debilitating disease in humans and potentially significant damage to livestock and food supplies, *Brucella* species are considered biological weapons of mass destruction (Horn and Breeze 1999, Kortepeter and Parker 1999). These biological warfare agents are listed as overlap select biological agents by the Department of Health and Human Services (HHS) (42 CFR 73; December 13, 2002) and the United States Department of Agriculture (USDA) (9 CFR 121 and 7 CFR 331; December 13, 2002). Of these select agents, the complete genomic sequences of *B. melitensis* and *B. suis* have been published (DelVecchio et al 2002, Paulsen et al 2002).

The outer membranes of brucellae are like that of other Gram-negative bacteria in that the outermost layer is lipopolysaccharide (LPS) (Cherwonogrodzky et al 1990). *Brucella* species which are smooth contain an LPS in which O-polysaccharide (OPS) molecules are attached to lipid A via core oligosaccharides. These species include *B. melitensis, B. suis, B. abortus,* and *B. neotomae* (Cherwonodrodzky et al 1990, Timony et al 1988). In contrast, rough *Brucella* species, *B. ovis* and *B. canis*, lack OPS (Cherwonogrodzky et al 1990). When OPS is removed from smooth LPS *Brucella* strains there is a significant decrease in virulence (Kreutzer and Robertson 1979); however, *B. canis* and *B. ovis*, which are both naturally occurring rough *Brucella* species are virulent in their natural hosts (Timony et al 1988).

Two antigens, A and M, are associated with the OPS of smooth *Brucella* species. First isolated from *B. abortus*, the A antigen consists of linear unbranched homopolymers of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units (perosamine), whereas the M antigen isolated from *B. melitensis* OPS consists of a linear unbranched chain with pentasaccharide perosamine repeating units containing one 1,3-linked and four 1,2-linked residues (Bundle et al 1987). The presence of the A or M antigen is used to distinguish between species and biovars of *Brucella*; however, in some strains these antigens appear simultaneously or may be absent altogether (Timony et al 1988). *Brucella* OPS is an immunogenic antigen, provoking the initiation of the host's humoral immune response (Hurvell 1973) and thus the formation of anti-OPS antibodies. Serological diagnosis of infected animals is based on the presence or absence of anti-OPS antibodies. Currently available serological tests for brucellosis are the Rose Bengal test, Plate Agglutination test, Card test, Standard Tube Agglutination test, Complement Fixation test, Milk Ring test, Coombs test, Rivanol test, and Enzyme-linked Immunosorbant Assay (ELISA) (MacMillan 1990, Timony et al 1988).

Brucella suis

In 1914, Jacob Traum first isolated *Brucella suis* from an aborted piglet found in an Indiana swine herd (Alton 1990). Since then, five biovars of *B. suis* have been defined and characterized based on specific growth requirements such as: utilization of serum, dye sensitivity, carbon dioxide requirement, hydrogen sulfide production, bacteriophage susceptibility, and the presence of the A and/or M antigens on the OPS (Meyer 1990). Biovar 1 displays the most classical characteristics of *B. suis* and is the most prevalent in the United States. The primary hosts of biovars 1, 2, and 3, are swine. Biovars 1, 2, and 3 are present in most swine-raising areas of the world, while biovar 4 infects reindeer and caribou in the Arctic Circle. All but biovar 2 have been associated with undulant fever in humans and are therefore zoonotic agents. Biovar 5, which is still poorly characterized, is believed to infect only murine species (Deyoe and Manthei 1975, Manthei 1968, Timony et al 1988).

Swine Brucellosis

Brucella suis causes swine brucellosis, which is manifested by abortion and infertility in females and males. Swine brucellosis is difficult to diagnose due to the number of asymptomatic cases, and disease is often not noted until there is a decrease in herd reproduction (Alton 1990). Abortion is the most common manifestation in females (Deyoe and Manthei 1975); however, piglets may be delivered healthy, stillborn, mummified, or weak. Mastitis, abscess formation, and chronic metritis are also common, with the latter being the major cause of infertility in sows (Alton 1990, Leman et al 1974). Brucellae are shed from the uterus for up to 30 months post-parturition, potentially infecting an entire herd (Deyoe and Manthei 1975).

Unlike *B. abortus* and *B. melitensis*, *B. suis* is a venereal disease and boars are susceptible to infection. In males, swelling and atrophy of the epididymides and unilateral or

bilateral orchitis are common with persistent genital infection resulting in the shedding of brucellae in the semen for up to three to four years after infection. Both sexes may experience lameness and swollen joints (Alton 1990, Deyoe and Manthei 1975, Enright 1990). Spondylitis results from the localization of brucellae in the lumbar and sacral regions of the vertebrae, and infection can also result in paralysis in some animals from pressure on the spinal cord due to necrotic tissue (Timony et al 1988).

The majority of *B. suis* infections occur via exposure of the alimentary or genital tracts (Deyoe and Manthei 1975). During ejaculation the boar produces a large amount of semen which migrates through the cervical folds into the sow's uterus, making venereal transmission fairly common (Manthei 1968). The consumption of feed that has been contaminated by urine or genital excretions could also provide a mode of entry for the organism (Timony et al 1988). Transmission may also occur via contact with the respiratory tract, conjunctiva, or skin (Deyoe and Manthei 1975).

The course of infection of *B. suis* is similar to that of all other *Brucella* species. The brucellae first penetrate mucosal membranes, typically the uterine, oral, or nasopharyngeal mucosa, in order to gain entry into the host. Once passage through mucosal membranes has occurred, brucellae are able to reside within host macrophages which transport the organism to draining lymphatics and regional lymph nodes (Enright 1990). As the organism begins to replicate, local inflammation develops and the lymph nodes become hemorrhagic. Access to the blood results in bacteremia, which, in the case of *B. suis* infection, is most persistent during the first eight weeks following infection, but may persist for years in swine displaying clinical symptoms (Deyoe and Manthei 1975). The incubation period of *B. suis* is approximately 10-30 days, after which the pig is considered to be infected (Manthei 1968), and in young piglets often

a latent infection sets in, impeding diagnosis prior to the immediate post-weaning period (Goode et al 1952). Swine infected after five months of age, the onset of sexual maturity, are more likely to develop chronic brucellosis than those infected as suckling or weanling piglets (Manthei et al 1952).

Brucella suis localizes to the reproductive tract, skeletal system, joints, mammary glands, lymph nodes, spleen, liver, kidneys, bladder, and brain. In the infected sow or gilt, chronic metritis typically develops which interferes with conception and contributes to the prolonged shedding of brucellae from the uterus. This also leads to late-gestation abortions, usually occurring at 65-72 days (Enright 1990, Leman et al 1974).

Vaccine Strains

Live vaccines for brucellosis, in general, provide more complete and lasting immunity than killed or subcellular vaccines. This is due to their ability to provoke cell-mediated immune responses which are necessary to clear intracellular bacterial infections such as *Brucella* (Schurig et al 2002). An ideal vaccine should be one that is stable; can be easily produced and stored; and provides lasting immunity. In addition, the vaccine should not induce immune responses that interfere with diagnostic tests and be non-pathogenic to vaccinated animals and humans handling the vaccine (Nicoletti 1990). Currently there is no commercially available vaccine for swine brucellosis, but current research efforts are underway.

***Brucella abortus* Strain 19.** Dr. John M. Buck, a bacteriologist with the Pathological division of the Bureau of Animal Industry, Washington, D.C., recovered a virulent strain of *B. abortus* from the milk of a Jersey cow in June 1923. After passaging and maintaining the culture at room temperature for one year the organism's virulence was lost, and *B. abortus* Strain 19, the nineteenth stock culture of the series, was tested as a potential vaccine for bovine brucellosis.

This vaccine was found to be protective for bovine brucellosis and introduced for field usage in 1941. Since then Strain 19 has become the most widely used cattle vaccine for brucellosis (Graves 1943, Nicoletti 1990). Strain 19, like the smooth strains *B. melitensis*, *B. abortus*, and *B. suis*, causes undulant fever in humans and therefore extreme care must be taken when handling this vaccine (Nicoletti 1990).

Strain 19 has proven to be an efficacious bovine vaccine due to its high antigenicity and low virulence (Lawson 1950, Schurig 2002 et al). Protection in vaccinated pregnant heifers after virulent *B. abortus* challenge was greater than 90% when compared to unvaccinated controls (Confer et al 1985, Nicoletti 1984). Calves administered one dose at approximately six months of age sustain immunity to virulent *B. abortus* for five or more pregnancies (McDiarmid 1957).

While Strain 19 is an efficacious vaccine, there are several problems associated with its use in cattle. The first issue lies within the fact that Strain 19 contains OPS similar to field strains of *B. abortus* (Lawson 1950), and vaccination with this strain leads to the production and persistence of OPS-specific antibodies (Nicoletti 1990). Current serological tests for brucellosis rely on the presence of these antibodies to distinguish infected from non-infected animals, therefore making it difficult to differentiate vaccinated from field strain infected cattle during routine brucellosis screening (Schurig et al 2002). It is estimated that 0.5-15% of Strain 19 vaccinates become persistent reactors to brucellosis diagnostic tests even when they receive a reduced dose of the vaccine (Alton et al 1980, Beckett and MacDiarmid 1987, Nicoletti 1984). It is strongly discouraged to use Strain 19 during pregnancy, when the animal is most susceptible to brucellosis infection, since it may induce abortion in 0.5-2.5% of vaccinates (Beckett and MacDiarmid 1987, Nicoletti 1990, Schurig et al 2002). While Strain 19 is an efficacious vaccine

in cattle, swine vaccinated SC with this vaccine were not protected against field exposure to virulent *B. suis* (Lindley and Lander 1949).

***Brucella suis* Strain 2.** In an effort to find a vaccine for swine brucellosis, *Brucella suis* Strain 2 was tested in China in the late 1970s. This vaccine is a laboratory adapted smooth strain of *B. suis* biovar 1 that was attenuated naturally after serial transfer on culture media for years. It has been shown to be efficacious when administered orally in sheep, goats, cattle, and swine. Pigs that received two oral doses of Strain 2, two to three months apart, were afforded 75% protection against conjunctival virulent *B. suis* infection without persisting antibody titers; however, no protection was seen when animals were challenged by mating with a boar excreting *B. suis* in his semen. This becomes problematic since venereal transmission of *B. suis* is common (Nicoletti 1990, Xin 1986). The dose of Strain 2 administered per animal has never been reported since the live culture was poured into water troughs and individual consumption was not monitored, and parenteral administration in ruminants resulted in abortion (Xin 1986).

***Brucella abortus* Strain RB51.** Schurig et al serially transferred *B. abortus* Strain 2308 onto tryptic soy broth agar (TSBA) plates supplemented with rifampin and penicillin. Prior to each transfer, single colonies displaying rough colony morphology and antibiotic resistance were isolated, and after a total of 51 transfers the rough attenuated *B. abortus* Strain RB51 was shown to be stable in BALB/c mice with no reversion to a smooth phenotype. Colonization experiments in mice demonstrated a decrease in colony-forming units (CFU)/spleen of the vaccine over time with no RB51 recovered at four weeks post-inoculation (p.i.). When 1×10^8 CFU of RB51 was inoculated intraperitoneally (IP) in mice seven weeks prior to challenge with virulent *B. abortus* Strain 2308, vaccinated mice had decreased numbers of CFU of strain 2308 in their spleens at one and four weeks post-challenge as compared to controls. At no point

during any vaccination protocol were anti-OPS antibodies present in sera of RB51 vaccinates prior to challenge (Schurig et al 1991). Further studies with pregnant mice infected with *B. abortus* strains showed less placental damage and colonization with RB51 than with Strains 19 and 2308 (Tobias et al 1992). Mice vaccinated IP or subcutaneously (SC) with RB51 showed increased protection against virulent challenge with *B. abortus, B. melitensis,* and *B. ovis* (Jimenez de Bagues et al 1994).

Experiments performed in RB51 vaccinated cattle have shown that the vaccine is cleared from draining lymph nodes at a rapid rate (Cheville et al 1992). The clinical disease and pathologic lesions of acute brucellosis are not caused by RB51 (Cheville et al 1996). These observations were supported with research conducted by Palmer et al in 1997, which determined that RB51 vaccination in pregnant cattle is non-abortogenic and both humoral and cell-mediated immune responses are stimulated without infection of fetal or maternal tissues (Palmer et al 1997b). Calves vaccinated SC with RB51 at three to ten months of age were protected from virulent *B. abortus* infection at levels comparable to that of Strain 19; therefore, RB51 was named the official calfhood vaccine for bovine brucellosis by the Animal and Plant Health Inspection Service, United States Department of Agriculture in 1996 (9 CFR 78; August 12, 1996). In 2004, RB51 received full USDA licensure as a brucellosis vaccine for cattle and bison.

Due to the high prevalence of brucellosis in wild ungulates, RB51 was evaluated as an oral vaccine candidate. Oral vaccines are used to vaccinate large numbers of a target population. Mice and cattle were used as models for the oral administration of RB51. Oral vaccination provided less protective immunity than IP administration against virulent *B. abortus* challenge in BALB/c mice (Stevens et al 1996). Cattle were afforded protection from virulent *B. abortus* infection by oral vaccination with RB51. Oral vaccinates had a 30% abortion rate and 20% of

these animals were colonized by virulent *B. abortus*; however, at no time was RB51 isolated from vaccinates or their calves (Elzer et al 1998). The oral administration of RB51 has also been evalutated in dogs and found to be non-pathogenic (Palmer et al 1997a).

RB51 was evaluated as a heterologous vaccine due to the protection it afforded cattle against virulent *B. abortus* challenge. Subcutaneous administration of RB51 in swine resulted in 100% colonization of draining lymph nodes. Partial protection against virulent *B. suis* challenge, as well as increased litter size and fewer dead piglets compared to controls were found in RB51 vaccinated sows (Hagius et al 1995). Lord et al determined in 1998 that swine were protected from virulent *B. suis* challenge by intramuscular (IM) or oral administration of RB51 regardless of the age of the pigs at vaccination, the vaccine dose, or the number of doses given. Edmonds et al further evaluated RB51 as a potential oral vaccine for swine in 2001, utilizing a viscous liquid (Karo syrup) and a scarification mechanism (pecans) which both prolonged adherence and allowed a route of entry for the vaccine via the oral mucosa. Concentrations of greater than 1 x 10^{10} CFU of RB51 resulted in 100% colonization of the vaccine strain in draining lymph nodes when this method was used.

***Brucella suis* Strain VTRS1.** In 1996 Virginia Polytechnic University introduced a genetically attenuated strain of *Brucella suis* Strain 2579 (biovar 4) as a potential heterologous vaccine for brucellosis. This rough mutant, *B. suis* Strain VTRS1, was produced via allelic exchange, in which the *rfbU* gene that encodes for OPS expression was disrupted and replaced with a kanamycin resistance gene to allow for isolation. There was no reversion to the smooth phenotype observed during *in vivo* passage in BALB/c mice, and the vaccine did not induce the production of anti-OPS antibodies. VTRS1 replicated in mice and offered substantial protection

against virulent challenge with *B. melitensis, B. abortus,* and five field strains of *B. suis* (Winter et al 1996).

In a small pilot study, VTRS1 was shown to transiently colonize swine and was not abortogenic when given conjunctively to pregnant sows. Subcutaneous vaccinates challenged with virulent *B. suis* at mid-gestation displayed a 0% abortion rate. The challenge strain was not recovered from vaccinated sow or piglet tissues and at no time were OPS-specific antibodies detected in the sera of vaccinates prior to challenge (Hagius et al 2001).

Pseudorabies Virus

Pseudorabies virus (PRV), or Aujeszky's Disease, was first described in 1902. Dr. Aladar Aujeszky, a Hungarian physician and veterinarian, recovered the virus from a dog, cat, and ox, and discovered that it was readily transmissible to rabbits and guinea pigs. Pseudorabies virus is an alphaherpesvirus approximately 180 nanometers in diameter. The virion consists of a double-stranded DNA genome, an icosahedral capsid made up of 162 capsomeres, and an outer membrane, or envelope (Gustafson 1981, Hanson 1954).

It is speculated that PRV has been present in the United States since 1813, and it is currently present in all of Europe, North, Central, and South America, India, Southeast Asia, Taiwan, North Africa, and New Zealand. This potentially devastating virus has an extremely broad host range, infecting and causing death in most mammals; however, swine are particularly resistant to infection and act as a reservoir for PRV. So far there have been no reports of human infection (Gustafson 1981).

The symptoms of PRV in swine vary from subclinical to death, with cardinal signs including fever, depression, excessive salivation, and convulsions (Gustafson 1981), and some strains of PRV will produce a respiratory disease (Lee and Wilson 1979). The mortality rate of

mature swine with a PRV infection is approximately two percent (Gustafson 1981); however, mortality is indirectly proportional to age, and therefore PRV is 100% fatal in piglets less than seven days old (Aiello 1998).

Pseudorabies virus infection of a pregnant sow may lead to infection of fetuses *in utero*, and can result in abortion, stillborn, mummified, and/or macerated piglets (Lee and Wilson 1979). If infection occurs during the first 30 days of gestation the embryos are typically reabsorbed, while from 40 to 60 days gestation, infection results in the death of some or all of the fetuses and/or premature expulsion. After 80 days gestation, macerated, stillborn, and weak piglets often result from PRV infection of the sow (Gustafson 1981, Iglesias 1993). Twenty percent of infected sows will not conceive at the next breeding (Gustafson 1981).

In domestic swine herds PRV is most commonly transmitted by inhalation or ingestion (Gustafson 1981). The virus can survive in wood, hay, and food for up to 46 days (Lee and Wilson 1979) and can persist for up to seven hours in air and travel via aerosols for up to two kilometers in certain weather conditions (Aiello 1998); therefore, swine housed in the same air space as infected animals may become infected even without direct contact (Iglesias 1993). All animals are potential lifetime carriers of PRV, and dogs, cats, and feral animals can transmit the virus between farms prior to death (Aiello 1998, Iglesias 1993).

Feral swine have been shown to primarily transmit PRV to one another through sexual contact, as with *B. suis*. Latent infections have been found primarily within the sacral ganglia in feral swine, as compared to the trigeminal ganglia localization in domestic swine, and feral boars do not typically transmit the virus to other boars, even when housed together (Romero et al 2001). This evidence, along with the isolation of virus from genital swabs, but not nasal swabs

of feral swine (Hahn et al 1997), indicates that PRV transmission in feral swine is primarily venereal.

Pseudorabies virus infection can result in both chronic and latent persistence (Gustafson 1981) with viral dissemination occurring via the central nervous system, the lymphatics, and the vascular system (Lee and Wilson 1979). The primary sites of viral replication are the nasal pharyngeal and tonsillar epithelium, depending on the mode of entry of the virus. The virus then moves to both the regional lymph nodes and the brain and continues replication. Within two to five days post-infection viral excretion begins, after which live virus can be recovered from nasal secretions, tonsillar epithelium, vaginal and preputial secretions, milk and urine (Aiello 1998).

There is currently no treatment for PRV and no vaccine available that will prevent infection, and while both a live attenuated vaccine and an inactivated vaccine are commercially available, they may interfere with diagnostic tests (Gustafson 1981). As with *B. suis*, research is currently underway to develop an efficacious vaccine for PRV that will enable swine herd owners and veterinarians to differentiate infected animals from vaccinates.

Pseudorabies Virus Glycoproteins

Ten PRV glycoproteins have been identified and characterized to date: gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM (Mettenleiter 1996). These glycoprotein spikes project from the virion's surface and are involved in attachment to host cells, fusion with the cellular membrane, entry of the nucleocapsid, and mediation of virus spread from infected to uninfected cells (Mulder et al 1997). The presence of gC, gE, gG, gI, or gM is not essential for infectivity of the virus; however, the absence of gB, gD, gH, gK, or gL prevents productive replication (Mettenleiter 1996).

Glycoprotein D is approximately 50-60 kilodaltons (Mettenleiter 1991), and its interaction with an unidentified receptor mediates the stable attachment of the virion and penetration into the host cell; however, it is not required for the subsequent cell-to-cell transmission of PRV. In cells that express PRV gD the virus can bind but penetration is inhibited, thus these cells are resistant to infection (Hanssens et al 1995, Mettenleiter 1991, Mulder et al 1997).

Herpesvirus glycoproteins are the principle antigens that induce host immune responses (Ishii et al 1988). Several studies have shown that gE, gC, and gD possess epitopes that induce the production of neutralizing antibodies and are the major targets of immune responses such as antibody and complement mediated cell lysis, antibody-dependent cell-mediated cytotoxicity, and cytotoxic T lymphocytes (Ishii et al 1988, Mulder et al 1997). Antibodies against gD are the most potent for virus neutralization in the absence of complement and inhibit virus penetration, thereby blocking the first stages of PRV infection (Eliot et al 1988, Mettenleiter 1991, Mettenleiter 1996).

Glycoprotein D has at least one epitope that induces the synthesis of virus neutralizing antibodies, making it a potential candidate for a useful subunit vaccine. In mice, gD has been shown to initiate the formation of virus neutralizing antibodies, and therefore may be as effective in swine (Ishii et al 1988). When gD was delivered to mice via infection with recombinant vaccinia virus or as a subunit synthesized in a eukaryotic cell line, PRV-induced mortality was prevented. In addition, gD that was synthesized in a Chinese Hamster Ovary cell line prevented lethal PRV infection in swine (Marchioli et al 1987). Vaccination of pigs with plasmid DNA encoding gD has been shown to stimulate humoral immune responses and protect against virulent challenge with PRV. The virus was shed from vaccinates for a shorter period of time

and a decrease in severity of clinical symptoms was observed (Haagmans et al 1999). A eukaryotic expression vector encoding gD has also been evaluated as a potential PRV vaccine. When administered to mice via live *Escherichia coli*, this subunit vaccine elicited cell-mediated immune responses and partially protected against lethal PRV challenge (Shiau et al 2001).

In diagnostic tests for PRV that are currently available and used for the screening of swine herds, the presence of antibodies to proteins other than gD interfere with the differentiation between infected swine and vaccinates (Marchioli et al 1987). The use of gD as a vaccine for PRV in swine would eliminate this uncertainty. This advantage, coupled with the fact that gD is the target of neutralizing antibodies that can protect mice and swine from PRV disease, make gD the perfect candidate for an effective subunit vaccine.

Feral Swine

Feral swine (*Sus scrofa*) are wild-living descendents of domestic swine that interbred years ago with the Eurasian wild boar, and were introduced into the United States by European immigrants during colonial times. The population of free-living feral swine in the United States is now estimated at over two million (Conger et al 1999) spread out over at least 32 states (Romero 2001).

The overall incidence of *B. suis* and PRV in feral swine is estimated at 10-25% and 60%, respectively (Conger et al 1999, Hahn et al 1997). The mixing of feral and domestic pigs, and the feeding of offal and aborted fetuses to domestic pigs allows for the transmission and perpetuation of these diseases (Hahn et al 1997). Farmers that introduce feral swine into their domestic herds are 2.8 times more likely to also introduce *B. suis*, and the risk of the herd contracting PRV also increases by 8.9 times. Due to its potential airborne transmission, simply

having feral swine in the same neighborhood as a domestic herd increases the chances of contracting PRV by 3.1 times (Dees 1999).

Since the reservoirs of *B. suis* and PRV are primarily feral swine, this population should be the target of vaccination programs, and considering the nature of the recipients, oral administration would provide the easiest mode of vaccination. The most efficacious vaccine for *B. suis*, VTRS1, combined with the most immunogenic subunit of PRV, gD, could quite possibly offer a multivalent vaccine for both diseases that could be administered orally to the feral swine population.

The goal of this project is to evaluate the use of the rough attenuated strains RB51 and VTRS1 as oral vaccines for swine brucellosis, and to determine whether the addition of a plasmid encoding for the PRV gD stimulates immune responses to rough *Brucella* antigens and gD while transiently colonizing lymph node tissues. Future research evaluating the efficacy of these multivalent vaccines against pseudorabies in swine is necessary; however, it is hoped that these vaccines may ultimately assist in brucellosis and pseudorabies eradication programs by targeting the feral swine population.

CHAPTER 3: MATERIALS AND METHODS

Brucella Strains

B. abortus Strain RB51 and *B. suis* Strain VTRS1 were used as vaccine strains, and *B. suis* biovar 1 Strain 1330 was used as the challenge strain. Additionally, *B. abortus* Strain 2308 cell lysates were used for immunological assays. Stocks of RB51 *B. suis* 1330, and *B. abortus* 2308 were plated onto Schaedler blood agar (SBA) plates, and VTRS1 was plated onto SBA plates supplemented with 45 ug/ml kanamycin. Plates were incubated at 37°C, 5% CO_2, for 48-72 hours. Bacterial lawns were harvested with *Brucella* broth and turbid metrically aliquoted into 1.5 ml cryovials containing 1 x 10^9, 1 x 10^{10}, or 1 x 10^{11} CFU/ml. These infectious doses were snap frozen in liquid nitrogen and stored at –80°C. Serial dilutions of each strain were plated after preparation and prior to each vaccination or challenge to determine viable counts of bacteria.

Animals and Breeding

Pigs were purchased from the Louisiana State University AgCenter Swine Unit, Baton Rouge, LA, or the Wade Correctional Center, Homer, LA, both of which are certified brucellosis-free facilities. The swine were housed at the Ben Hur Large Animal Isolation Facility, a USDA/APHIS/VS/CDC approved facility, provided with water *ad lib*, and fed ground corn. Prior to vaccination all swine were deemed *Brucella* antibody-negative by the *Brucella* Card test and Western immunoblot analysis. Prior to breeding gilts were administered 7 ml of Regu-Mate ® (altrenogest) Solution 0.22% (2.2 mg/ml) (DPT Laboratories, San Antonio, TX) orally for 14 days in order to synchronize their estrus cycles. Three to six days later gilts were individually bred with a *Brucella*-negative boar. Pregnancy was confirmed via ultrasound. All

animals were treated humanely in accordance with LSU AgCenter Animal Care and Use Committee guidelines.

Genetic Manipulation of Vaccine Strains

The PRV gD antigen was incorporated into RB51 and VTRS1 via the plasmid pBBgroE ssLgD-CM^R, obtained from Purdue University. One hundred ul of 1 x 10^9 CFU/ml RB51 or VTRS1 was streaked onto SBA plates, grown for 2-3 days at 37°C, 5% CO_2, and harvested with sterile *Brucella* broth (Difco Laboratories, Detroit, MI). The harvested bacteria were used to inoculate 25 ml of sterile *Brucella* broth which was incubated in a 37°C shaking water bath for 24 hours. The culture was centrifuged for 5 minutes at 15,000 rpm, the supernatant was discarded, and the pellet was re-suspended in 1 ml cold dH_2O. The pellet was then washed three times with 1 ml cold dH_2O, and the final pellet was re-suspended in 100 ul cold dH_2O. Thirty-three ul of cold suspension and 5 ul of cold plasmid solution were combined into an ice cold 2 mm, 400 ul Eppendorf Electroporation Cuvette (Brinkmann Instruments, Inc., Westbury, NY). This solution was electroporated at 2.5 kV using an Eppendorf Electroporator 2510 (Brinkmann Instruments, Inc., Westbury, NY). Five hundred ul of cold SOC-B (6% trypticase soy broth, 10 mM NaCL, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (Lai et al 1990) recovery media was added to the solution, and the final culture was incubated in a 37°C shaking water bath overnight. One hundred ul of the recovered culture was spread on SBA plates supplemented with 30 ug/ml chloramphenicol. Plates were incubated at 37°C, 5% CO_2 and monitored daily. Resultant colonies were patched onto SBA plates supplemented with 30 ug/ml chloramphenicol and incubated at 37°C, 5% CO_2, overnight, after which they were inoculated into 5 ml *Brucella* broth supplemented with 30 ug/ml chloramphenicol and incubated overnight in a 37°C shaking water bath.

Plasmid minipreps were performed on colonies to confirm the presence of pBBgroE ssLgD using the Qiagen Buffer System (Qiagen, Inc., Valencia, CA). After centrifuging 1.5 ml of RB51+gD or VTRS1+gD culture at 9000 rpm for 5 minutes and discarding the supernatant, the pelleted cells were resuspended in 300 ul of 100% chloroform and 300 ul P1 buffer (50 mM Tris base, 10 mM EDTA, 10 ug/ml RNaseA). This suspension was centrifuged at 15,000 rpm for 5 minutes, after which the aqueous and cell layers were transferred to a clean tube, combined with 300 ul of P2 buffer (200 mM NaOH, 1% SDS), and mixed by gently inverting the tube. Three hundred ul of chilled P3 buffer (3.0 M CH_3CO_2K) was then added and the suspension was again gently mixed by inverting the tube. After centrifuging the solution at 14,000 rpm for 10 minutes, the supernatant was placed into a clean tube. Plasmid DNA was precipitated with 630 ul of isopropanol and the suspension was centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed and the pellet was allowed to dry. The pellet was re-suspended in 25 ul 1X TE (1 mM Tris, 0.1 mM EDTA) and incubated at 4^oC for 1 hour. The presence of plasmid DNA was verified via agarose gel electrophoresis by running 10 ul of plasmid DNA on a 0.7% agarose gel at 90 V for 1 hour. After staining with ethidium bromide, the presence of a band at 7.2 Kb confirmed the presence of pBBgroE ssLgD-CM^R. Once the plasmids were confirmed, 25% glycerol stocks and 1 x 10^9 and 1 x 10^{11} CFU/ml infectious doses were made of RB51+gD and VTRS1+gD.

Vaccine Routes

Pigs were vaccinated either orally or SC. For SC vaccination, 1 x 10^{10} CFU of each vaccine was inoculated into the right cervical area using a 3 ml syringe with a 20 gauge, 1 inch needle. Oral vaccines were prepared by adding a 1 x 10^{11} CFU/ml infectious dose of each strain to 9 ml sterile injectable saline and 10 ml Karo syrup, and 3 ml of the resultant mixture was

poured over a 2:1 mixture of pecan shells to cracked corn. This was fed to each pig individually to ensure that each animal received greater than 1 x 10^{10} CFU of the vaccine. Controls received 3 ml of a 1:1 mixture of Karo syrup to sterile injectable saline. To facilitate oral mucosa exposure, Karo syrup, which prolongs the adherence of the brucellae, and pecan shells were used to scarify the mucosa (Edmonds 2001).

Serum Collection

Pigs were bled from the brachiocephalic vein using an 18 gauge, 1.5-inch needle and 20 ml syringe (Lawhorn 1988). Sera were separated via centrifugation and stored at –20^{o}C until tested.

Serological Testing

All sera were tested by the Card test and Western immunoblot analysis as previously described (Edmonds et al 2001). Briefly, for Western immunoblots, glycerol stocks of *Brucella* strains were plated onto SBA plates and grown for 2-3 days at 37^{o}C, 5% CO_2. Cells were harvested with Phosphate Buffered Saline (PBS), sonicated for 8 minutes, and boiled for 10 minutes. The resultant cell lysates were diluted 1:1 with Laemmli sample buffer (BioRad Laboratories, Hercules, CA) and boiled an additional 10 minutes. VTRS1+gD and RB51+gD were plated onto SBA plates supplemented with 30 ug/ml chloramphenicol and incubated at 37^{o}C, 5% CO_2, for 3-4 days. Cells were harvested and used to inoculate 5 ml of *Brucella* broth supplemented with 30 ug/ml chloramphenicol and incubated in a 37^{o}C shaking water bath overnight. The bacteria were then heat shocked in a 42^{o}C shaking water bath for 30 minutes to increase plasmid copy number, then centrifuged for 5 minutes at 15,000 rpm. The supernatant was discarded and cells were re-suspended in 2 ml dH_2O. Suspensions were sonicated and antigens prepared as described above. Various concentrations of cell lysates were separated by

polyacrylamide gel electrophoresis (PAGE) using 12% Tris-HCl BioRad Ready Gels (BioRad Laboratories, Hercules, CA) at 150 V for 45 minutes. Proteins were transferred onto nitrocellulose paper at 100 V for 1 hour. The nitrocellulose was then blocked with 5% non-fat milk, washed five times with Tris-Buffered Saline (TBS)-Tween and once with TBS (0.5 M NaCl, 20 mM Tris). Blots were individually incubated overnight at room temperature on a shaker with a 1:40 dilution of serum to be tested. After another washing cycle, blots were next incubated for 45 minutes at room temperature on a shaker with a 1:800 dilution of anti-pig IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO). Blots were developed after a third washing cycle with 4-chloro-1-naphthol (Sigma-Aldrich Co., St. Louis, MO) and allowed to dry.

Necropsy

Pregnancies were monitored until delivery and the numbers of live, stillborn, or aborted piglets were recorded. Piglets were euthanized by CO_2 asphyxiation if necessary, and samples of lung tissue and stomach fluid were taken from all piglets. Upon completion of the study, sows were euthanized at a USDA-inspected slaughterhouse by captive-bolt and exsanguination. Blood samples were collected and serological analyses were performed as described above. The following tissues were collected: parotid, prescapular, internal iliac, and supramammary lymph nodes; mammary gland; liver; spleen; and uterine swab.

Bacteriological Analysis

Tissue samples were weighed and homogenized in 20 ml PBS. One hundred ul of each sample was plated onto SBA plates supplemented with *Brucella* Selective Supplement (Oxoid Ltd., Basingstoke, Hampshire, England). Plates were incubated at 37^{o}C, 5% CO_2, for 14 days, after which colonies were counted and CFU/gram of tissue was calculated. Uterine swabs were streaked onto SBA plates supplemented with *Brucella* Selective Supplement and incubated at

37°C, 5% CO_2, for 14 days and recorded as positive or negative based on the presence or absence of colonies. RB51+gD or VTRS1+gD colonies were patched onto SBA plates supplemented with 30 ug/ml chloramphenicol or 45 ug/ml kanamycin and 30 ug/ml chloramphenicol, respectfully, to ensure that the plasmid was still present.

Experimental Design

Serologic Responses. Twelve sexually immature pigs (2-3 months old) were randomly placed into two groups: six RB51 vaccinates and six VTRS1 vaccinates. All swine were orally vaccinated with greater than 1 x 10^{10} CFU of the vaccine each day for three consecutive days at 0, 4, 6, and 8 weeks. Serum was obtained prior to vaccination and ten days following each vaccine exposure, and analyzed as described above.

Colonization. Fifty-two sexually immature pigs (2-3 months old) were randomly assigned into vaccination groups as follows: nine SC vaccinated with RB51+gD, nine SC vaccinated with VTRS1+gD, seven orally vaccinated with RB51+gD, seven orally vaccinated with VTRS1+gD, ten SC vaccinated with VTRS1 and ten orally vaccinated with VTRS1. Subcutaneous vaccinates received 1 x 10^{10} CFU once, while oral vaccinates received greater than 1 x 10^{10} CFU each day for three consecutive days. Two to four pigs from each group were euthanized at either day 10, 24, or 31, post vaccination. Samples of the parotid, prescapular, and internal iliac lymph nodes, liver, and spleen were collected for bacteriological analysis. Serum was obtained prior to vaccination and at necropsy, and analyzed as described above.

Pathogenesis. Seventeen pregnant sows were obtained and randomly assigned into three groups. At mid-gestation two groups of five sows were challenged with 1 x 10^{9} CFU of either RB51+gD or VTRS1+gD in 100 ul of PBS by administering half of the inoculum into the conjunctival sac of each eye. Since the vaccines are rough and thought to be semi-attenuated,

these groups received two logs greater CFUs as compared to the smooth virulent control strain. As positive controls seven sows were challenged with 1 x 10^7 CFU of the virulent strain *B. suis* 1330 conjunctivally. At parturition lung tissue and stomach fluid of live piglets that were euthanized with CO_2, aborted, and still-born piglets were collected then cultured. One month post-parturition all sows were euthanized and the following tissues were collected for bacteriological analysis: parotid, prescapular, internal iliac, and supramammary lymph nodes; mammary gland; liver; spleen; and uterine swab. Serum was obtained prior to challenge and at necropsy, and processed as described above.

Vaccine Efficacy. Forty-eight eight-month-old gilts were randomly assigned into groups: ten VTRS1 oral vaccinates, six RB51 oral vaccinates, ten VTRS1+gD SC vaccinates, ten VTRS1+gD oral vaccinates, five RB51+gD oral vaccinates, and seven saline controls. Swine were vaccinated orally with greater than 1 x 10^{10} CFU each day for three days or once subcutaneously with 1 x 10^{10} CFU. Four weeks post-vaccination, all orally vaccinated swine were boosted at two-week intervals in the same manner for a total of four exposures to the vaccines. Subcutaneous vaccinates were boosted once four weeks post-vaccination for a total of two exposures to the vaccines. Four weeks following the final boost the gilts were naturally serviced. At mid-gestation all swine were challenged with 1 x 10^7 CFU of the virulent strain *B. suis* 1330 in 100 ul of PBS by administering half of the inoculum into the conjunctival sac of each eye. At parturition lung tissue and stomach fluid of live piglets that were CO_2 euthanized, aborted, and still-born piglets were collected for culture. One month post-parturition all sows were euthanized and samples of the following tissues were collected for bacteriological analysis: parotid, prescapular, internal iliac, and supramammary lymph nodes; mammary gland; liver;

spleen; and uterine swab. Serum was obtained prior to vaccination, ten days following the final boost, prior to challenge, and at necropsy, and was tested as described above.

CHAPTER 4: RESULTS

Transformation of VTRS1 and RB51 with pBBgroE ssLgD

The parent strains VTRS1 and RB51 were electroporated with pBBgroE ssLgD, which encodes for the PRV gD protein via the cloning vector pBBR1MCS. This vector has been shown to be stably maintained extrachromasomally at a low copy number in both *B. abortus* and *B. suis* (Elzer et al 1995, Kovach et al 1994). Following transformation, plasmid minipreps were performed on colonies displaying chloramphenicol resistance, and the presence of plasmid DNA was visualized via agarose gel electrophoresis. Positive colonies contained plasmid DNA that migrated at approximately 7.2 kilobases (Kb) (Figure 1). In order to evaluate the growth of the resultant vaccines, VTRS1+gD and RB51+gD, 1 x 10^9 CFU/ml infectious doses of these strains as well as VTRS1 and RB51 were serially diluted and plated onto either SAB, SAB supplemented with *Brucella* Selective Supplement, or SAB supplemented with 30 ug/ml chloramphenicol. While the parent strain colonies grew after three to four days of incubation, VTRS1+gD and RB51+gD colonies required an additional 48 hours for growth. Plate counts of the transformed strains did not differ significantly from those of the parent strains (data not shown).

Serological Responses in Swine Orally Vaccinated with VTRS1 and RB51

Sexually immature pigs in each group were orally vaccinated with either RB51 or VTRS1 at weeks 0, 2, 4, and 6, and serological responses to rough *Brucella* antigens were determined via Western immunoblot analysis using RB51 and VTRS1 cell lysates ten days following each vaccination.

Fifty percent of RB51 vaccinates and 16.7% of VTRS1 vaccinates had a positive serological response to rough *Brucella* antigens after the first oral exposure. As expected, the

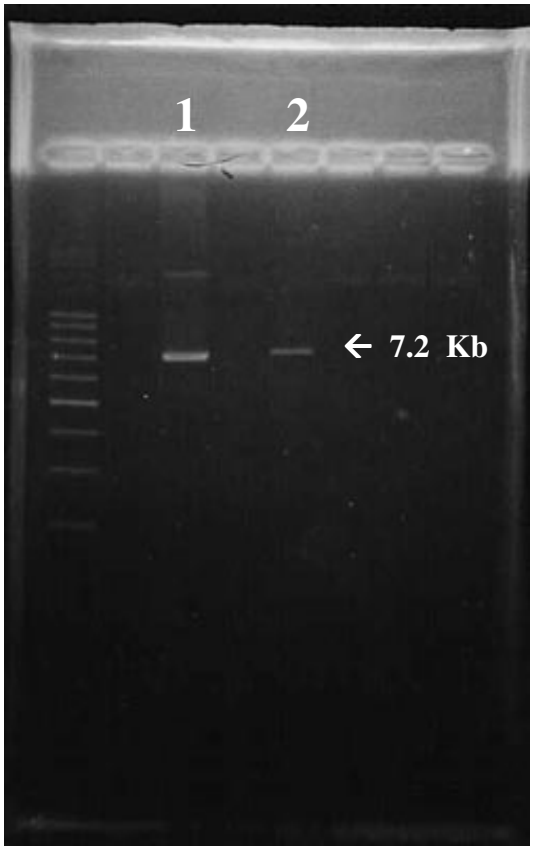

Figure 1 – Agarose gel electrophoresis of pDNA recovered from transformed vaccine strains. Lane 1: VTRS1+gD pDNA; Lane 2: RB51+gD pDNA.

number of seropositive pigs increased with each exposure to the vaccine, with 100% of RB51 vaccinates and 83.3% of VTRS1 vaccinates seropositive for rough *Brucella* antigens after four exposures to the vaccines (Table 1). At no time during the study were any of the vaccinates seropositive for the OPS antigens of smooth *Brucella*, as evidenced by the Card test and Western immunoblot analysis using *B. abortus* Strain 2308 and *B. suis* Strain 1330 cell lysates.

Colonization of VTRS1

Sexually immature pigs were vaccinated either orally or SC with VTRS1 and necropsied at day 10, 24, or 31. Lymph nodes were collected for bacteriological analysis and serum was tested for antibodies to rough *Brucella* antigens via Western immunoblot analysis using VTRS1 cell lysates.

VTRS1 was isolated from the parotid, prescapular, or internal iliac lymph nodes, or the liver or spleen of eight of the 20 vaccinates (40%). Subcutaneous vaccinates necropsied at day 10 or 24 had the greatest number of colonized tissues, 20% and 15%, respectively. Ninety percent of SC vaccinates and 80% of oral vaccinates were seropositive for rough *Brucella* antigens at necropsy (Table 2), confirming immune response stimulation and therefore vaccine exposure. At no point during the study were any vaccinates seropositive for the OPS antigens of smooth *Brucella*, as evidenced by the Card test and Western immunoblot analysis using *B. suis* Strain 1330 cell lysates.

Colonization of VTRS1+gD and RB51+gD

Sexually immature pigs were vaccinated SC or orally with either VTRS1+gD or RB51+gD. On day 10, 24, or 31 post-vaccination, pigs were necropsied and lymph nodes collected for bacteriological analysis. Serum was also collected pre-vaccination and at necropsy

Table 1 – Serological responses of pigs orally vaccinated with RB51 or VTRS1

Vaccine strain	Dose*	No. of pigs	No. of serologic responders§			
			Exposure 1	Exposure 2	Exposure 3	Exposure 4
RB51	1×10^{10} (x3)	6	3	5	6	6
VTRS1	1×10^{10} (x3)	6	1	2	5	5

*CFU/pig; Pigs were given the oral vaccine three consecutive days for each exposure.
§Western immunoblot using strains RB51 and VTRS1 cell lysates performed ten days post-exposure.

Table 2 – Colonization and serological responses of pigs vaccinated subcutaneously or orally with VTRS1

Route[*]	Dose[**]	No. of pigs	Necropsy (dpv)[†]	No. of serologic responders[‡]	No. culture positive[§]	Culture positive tissues[a]
SC	1×10^{10}	3	10	2	2	3/15 (38-125)
SC	1×10^{10}	4	24	4	2	3/20 (59-182)
SC	1×10^{10}	3	31	3	1	1/15 (74)
Oral	1×10^{10} (x3)	3	10	2	1	1/15 (333)
Oral	1×10^{10} (x3)	4	24	3	1	2/20 (100-8333)
Oral	1×10^{10} (x3)	3	31	3	1	1/15 (258)

[*]SC vaccinates were exposed to VTRS1 once, while oral vaccinates were exposed three consecutive days.
[**]CFU/pig.
[†]Days post-vaccination.
[‡]Western immunoblot using Strain VTRS1 cell lysate.
[§]Tissues collected: parotid, prescapular, and internal iliac lymph nodes, liver, and spleen.
[a]Culture positive tissues/ total number of tissues collected per group (range of CFU per gram of tissue).

and tested by Western immunoblot analysis for the presence of antibodies to rough *Brucella* antigens and PRV gD.

The vaccine strain was recovered from two of the 16 sexually immature pigs exposed either SC or orally to RB51+gD (12.5 %) (Table 3). VTRS1+gD was cultured from eight of the 16 vaccinated pigs (50%) (Table 4). Both vaccine strains were isolated from the draining lymph nodes and secondary lymphatic tissue (liver, spleen, and internal iliac lymph node) and colonies were transferred to SBA plates supplemented with chloramphenicol to ensure that the plasmid was not lost in the host. All vaccinates, including those colonized with the vaccine strains, were seronegative for smooth *Brucella* at all time points, as evidenced by the Card test and Western immunoblot analysis using *B. abortus* Strain 2308 and *B. suis* Strain 1330 cell lysates.

Serological responses to rough *Brucella* antigens were observed in 88.9% of SC and 71.4% of oral RB51+gD vaccinates (Table 3), while the VTRS1+gD vaccine provided 77.8% and 100% positive rough *Brucella* serology when administered SC and orally, respectively (Table 4). A positive serological determination, as evidenced by Western immunoblot analysis using RB51 and VTRS1 cell lysates, indicates that the rough *Brucella* antigens were seen by the vaccinates' immune systems.

A serological response to PRV gD was also measured by Western immunoblot analysis using purified PRV gD and RB51+gD cell lysates. Three of the nine (33.3%) SC RB51+gD vaccinates were positive for gD serology, while six of the nine (66.7%) SC VTRS1+gD vaccinates were positive. Only one pig from each oral vaccine group was positive for PRV gD antibodies (Tables 3 and 4).

Table 3 – Colonization and serological responses of pigs vaccinated subcutaneously or orally with RB51+gD

Route[*]	Dose[**]	No. of pigs	Necropsy (dpv)[†]	No. of serologic responders[‡]	No. of serologic responders to gD[§]	No. culture positive[a]	Culture positive tissues[b]
SC	1×10^{10}	3	10	2	0	1	1/15 (1850)
SC	1×10^{10}	3	24	3	2	0	0/15
SC	1×10^{10}	3	31	3	1	0	0/15
Oral	1×10^{10} (x3)	2	10	1	1	0	0/10
Oral	1×10^{10} (x3)	3	24	2	0	0	0/14
Oral	1×10^{10} (x3)	2	31	2	0	1	3/10 (45-462)

[*]SC vaccinates were exposed to RB51+gD once, while oral vaccinates were exposed three consecutive days.
[**]CFU/pig.
[†]Days post-vaccination.
[‡]Western immunoblot using Strain RB51 cell lysate.
[§]Western immunoblot using purified gD and Strain RB51+gD cell lysate.
[a]Tissues collected: parotid, prescapular, and internal iliac lymph nodes, liver, and spleen.
[b]Culture positive tissues/ total number of tissues collected per group (range of CFU per gram of tissue).

Table 4 – Colonization and serological responses of pigs vaccinated subcutaneously or orally with VTRS1+gD

Route[*]	Dose[**]	No. of pigs	Necropsy (dpv)[†]	No. of serologic responders[‡]	No. of serologic responders to gD[§]	No. culture positive[a]	Culture positive tissues[b]
SC	1×10^{10}	3	10	2	2	0	0/15
SC	1×10^{10}	3	24	2	2	1	2/15 (54-727)
SC	1×10^{10}	3	31	3	2	3	3/14 (16-71)
Oral	1×10^{10} (x3)	2	10	2	1	0	0/10
Oral	1×10^{10} (x3)	3	24	3	0	2	2/15 (63-167)
Oral	1×10^{10} (x3)	2	31	2	0	1	2/15 (27-43)

[*]SC vaccinates were exposed to VTRS1+gD once, while oral vaccinates were exposed three consecutive days.
[**]CFU/pig.
[†]Days post-vaccination.
[‡]Western immunoblot using Strain VTRS1 cell lysate.
[§]Western immunoblot using purified gD and Strain VTRS1+gD cell lysate.
[a]Tissues collected: parotid, prescapular, and internal iliac lymph nodes, liver, and spleen.
[b]Culture positive tissues/ total number of tissues collected per group (range of CFU per gram of tissue).

VTRS1+gD and RB51+gD Pathogenesis in Pregnant Swine

Pregnant sows were challenged conjunctively with $1x10^9$ CFU of VTRS1+gD or RB51+gD, or $1x10^7$ CFU of virulent *B. suis* at mid-gestation. Two logs greater CFU of the vaccines were used due to their rough morphology and attenuated nature. At parturition the numbers of live, stillborn, and aborted piglets were recorded, and samples of lung tissue and stomach fluid collected for bacteriological analysis. Four weeks post-parturition the sows were necropsied and bacteriological analysis of lymph nodes was performed. Serum was collected prior to challenge and at necropsy and tested via the Card test and Western immunoblot analysis.

The average litter size of sows challenged with VTRS1+gD was 11.4 piglets, 86% of which were born live, and RB51+gD challenged sows yielded an average litter size of 11.5 piglets and a live birth rate of 87%. The vaccine strains were not recovered from any of the piglet or sow tissues cultured thus far, while all of the control sows challenged with virulent *B. suis* and their litters were culture positive for the challenge strain (Table 5). Two RB51+gD challenged sows have not yet farrowed, and four other sows have not yet been necropsied. This study is ongoing and tissues from the remaining pigs will be evaluated once collected.

Efficacy of Oral VTRS1 and RB51 Vaccination

Gilts were vaccinated orally with VTRS1, RB51, or saline for three consecutive days at weeks 0, 4, 6, and 8, and then bred with a *Brucella* negative boar four weeks following the final boost. At mid-gestation all gilts were challenged conjunctively with 1×10^7 CFU of virulent *B. suis*, and pregnancies were monitored until delivery. At parturition the numbers of live, stillborn, or aborted piglets were recorded and samples of lung tissue and stomach fluid were collected for bacteriological analysis. Sows were necropsied four weeks post-parturition and bacteriological analysis of lymph nodes was performed. Serum was analyzed via the Card test and Western

Table 5 – Delivery status and culture results for sows challenged at mid-gestation with VTRS1+gD, RB51+gD, or virulent B. suis

Group	No. of pigs	Challenge Dose[*]	No. of live piglets	No. of dead piglets	No. of litters culture positive[†]	No. of sows culture positive[§]	Tissues culture positive[a]
VTRS1+gD	5	1×10^9	49	8	0/5	0/4	0/21
RB51+gD	5	1×10^9	40	6	0/3	0/2	0/14
B. suis	7	1×10^7	39	5	4/4[‡]	6/6	30/42 (30-11,550)

[*]CFU/pig; due to the rough morphology and attenuated nature of the vaccine strains, these groups received two logs greater CFU than pigs challenged with smooth virulent *B. suis*.
[†]Tissues collected from piglets: lung tissue and stomach fluid.
[‡]One saline control sow reabsorbed her fetuses shortly after challenge and another farrowed too close to the challenge date; therefore, these two litters were excluded from the study.
[§]Tissues collected from sows: parotid, prescapular, supramammary, and internal iliac lymph nodes, mammary gland, liver, spleen, and uterine swab.
[a]Culture positive tissues/ total number of tissues collected per group (range of CFU per gram of tissue).

immunoblot analysis prior to vaccination, ten days following the final boost, prior to challenge, and at necropsy. Three gilts, two RB51 vaccinates and one VTRS1 vaccinate, failed to conceive and were therefore excluded from the study.

Eight of the nine (89%) VTRS1 vaccinates were seropositive for rough *Brucella* antigens prior to virulent *B. suis* challenge as evidenced by Western immunoblot analysis using RB51 and VTRS1 cell lysates, while only three of the four (75%) RB51 vaccinates were positive. No control sows were seropositive for rough *Brucella* antigens (Table 6), and at no point prior to challenge were antibodies specific for the OPS antigens of smooth *Brucella* detected in any of the gilts, as determined via the Card test and Western immunoblot analysis using *B. abortus* 2308 and *B. suis* 1330 cell lysates.

The average litter size of sows vaccinated with VTRS1 was nine piglets, 93.8% of which were born live. The challenge strain was recovered from either lung tissue or stomach fluid of three of the nine (33%) litters and six of the nine (67%) sows were culture positive for virulent *B. suis* four weeks post-parturition (Table 6). The challenge strain was present in the primary and secondary lymph nodes and the spleen of the majority of culture-positive sows, but was also recovered from the mammary gland and liver of three sows and one sow's uterine swab (Table 7).

RB51 vaccinates yielded an average litter size of eight piglets. One sow aborted her litter of 11 piglets at 14 weeks gestation, six weeks after challenge; however, she was in poor health due to an abscess on her right hind leg and the challenge strain was not recovered from the aborted fetuses. The remaining piglets born to RB51-vaccinated sows (64%) were born live. The challenge strain was not cultured from the lung tissue or stomach fluid of any of the litters; however, 75% of the sows were culture positive for virulent *B. suis* four weeks post-parturition

Table 6 – Delivery status and culture results for swine vaccinated orally with VTRS1, RB51, or saline and challenged at mid-gestation with 1 x 10^7 CFU of virulent B. suis

Group	No. of pigs	No. of serologic responders[*]	No. of live piglets	No. of dead piglets	No. of litters culture positive[§]	No. of sows culture positive[a]
VTRS1	9	8/9	76	5	3/9	6/9
RB51	4	3/4	21	11[†]	0/4	3/4
Saline	7	0/7	39	5	4/4[‡]	6/6

[*]Western immunoblot using strains VTRS1 and RB51 cell lysates performed prior to challenge, after a total of four oral exposures to the vaccine or saline.
[†]One RB51 vaccinate aborted at approximately 14 weeks gestation, 6 weeks after challenge; however, she was in poor health due to an abscess on her leg and no piglets were culture positive for the challenge strain.
[§]Tissues collected from piglets: lung tissue and stomach fluid.
[‡]One saline control sow reabsorbed her fetuses shortly after challenge and another farrowed too close to the challenge date; therefore, these two litters were excluded from the study.
[a]Tissues collected from sows: parotid, prescapular, supramammary, and internal iliac lymph nodes, mammary gland, liver, spleen, and uterine swab.

Table 7 – Bacteriological analysis of tissues collected from sows orally vaccinated with VTRS1, RB51, or saline and challenged at mid-gestation with 1 x 10^7 CFU of virulent *B. suis*

	Vaccine Strains*		
	VTRS1	**RB51**	**Saline**
Litter Colonization			
Stomach Fluid	2/9† (22%) 0.5 (±1.04) ‡	0/4 (0%) 0§	4/4 (100%) 3.0 (±1.30)
Lung	1/9 (11%) 0.3 (±0.80)	0/4 (0%) 0§	3/4 (75%) 2.6 (±1.94)
Sow Colonization			
Parotid LN[a]	5/9 (56%) 1.3 (±1.28)	3/4 (75%) 2.4 (±1.70)	5/6 (83%) 2.3 (±1.36)
Prescapular LN	4/9 (44%) 0.9 (±1.14)	3/4 (75%) 2.4 (±1.68)	5/6 (83%) 2.0 (±0.98)
Supramammary LN	4/9 (44%) 1.1 (±1.51)	3/4 (75%) 2.2 (±1.51)	5/6 (83%) 2.2 (±1.23)
Mammary Gland	3/9 (33%) 0.9 (±1.39)	2/4 (50%) 1.6 (±1.98)	3/6 (50%) 1.1 (±1.18)
Liver	3/9 (33%) 0.7 (±1.09)	2/4 (50%) 1.8 (±2.07)	3/6 (50%) 1.0 (±1.07)
Spleen	4/9 (44%) 1.1 (±1.47)	3/4 (75%) 1.9 (±1.42)	6/6 (100%) 2.5 (±0.59)
Internal Iliac LN	4/9 (44%) 1.2 (±1.47)	2/4 (50%) 1.6 (±1.93)	3/6 (50%) 1.3 (±1.40)
Uterine Swab	1/9 (11%)	0/4 (0%)	2/6 (33%)
Card Test	8/9 (89%)	2/4 (50%)	4/6 (67%)

*Four oral exposures to 1 x 10^{10} CFU of VTRS1 or RB51, or saline; pigs were given the vaccine three consecutive days for each exposure.
†Number colonized/ number in treatment group.
‡Mean log CFU/gram of tissue.
§Limit of detection = 13 CFU/gram.
[a]LN = lymph node

(Table 6). The challenge strain was isolated from both primary and secondary lymph nodes, the liver and spleen, and the mammary gland of infected sows. Uterine swabs were all culture negative for virulent *B. suis* (Table 7).

Eighty-nine percent of the piglets born to saline control sows were live, with an average litter size of 7.3 piglets (Table 6). One sow reabsorbed her piglets shortly after challenge and another farrowed too close to the challenge date for her piglets to become infected. Both of these litters were excluded from the study; however, the sows were evaluated for colonization and serology post-parturition. All litters and sows were culture positive for the challenge strain (Table 6), with the lung tissue and stomach fluid of the piglets and the sows' primary and secondary lymph nodes and spleens containing the highest numbers of CFU per gram of tissue (Table 7).

Overall the litters of sows that were vaccinated orally with either VTRS1 or RB51 were significantly protected against infection with virulent *B. suis* (approximately three logs of protection as compared to saline controls). Oral vaccination with RB51 did not protect the sows; however, sows vaccinated orally with VTRS1 were afforded approximately one log of protection against virulent challenge as compare to saline controls (Table 7).

Evaluation of Oral and Subcutaneous Vaccination with VTRS1+gD and RB51+gD

Gilts were SC vaccinated with VTRS1+gD at weeks 0 and 4, or orally vaccinated with VTRS1+gD, RB51+gD, or saline for three consecutive days at weeks 0, 4, 6, and 8. These gilts are currently being bred with a *Brucella* negative boar and will be challenged conjunctively at mid-gestation with 1 x 10^7 CFU of virulent *B. suis*. At parturition the number of live, stillborn, and aborted piglets will be recorded and lung tissue and stomach fluid will be collected for bacteriological analysis. Sows will also be necropsied four weeks post-parturition and

bacteriological analysis of lymph nodes will also be performed. Serum was collected prior to vaccination and ten days following the final boost and analyzed via the Card test and Western immunoblot analysis using VTRS1, RB51, and RB51+gD cell lysates, and purified PRV gD. At necropsy serum will also be collected and tested. The serological analysis of pre-vaccination and post-vaccination sera will be discussed.

Subcutaneous vaccination with strain VTRS1+gD afforded the highest serologic response, with all vaccinates seropositive for rough *Brucella* antigens and 77.8% seropositive for PRV gD. Oral administration of VTRS1+ gD and RB51+ gD resulted in 70% and 60% of vaccinates mounting an immune response to rough *Brucella* antigens, respectively. Serologic responses to PRV gD in oral vaccinates were somewhat lower, with 20% of VTRS1+ gD vaccinates and 60% of RB51+ gD vaccinates positive for gD-specific antibodies (Table 8). At no point prior to challenge with virulent *B. suis* were any pigs shown to be positive for smooth *Brucella* anti-OPS antibodies, as determined by the Card test and Western immunoblot analysis using *B. abortus* Strain 2308 and *B. suis* Strain 1330 cell lysates.

Table 8 – Serological responses of pigs vaccinated with VTRS1+gD or RB51+gD

Vaccine strain	Route*	Dose**	No. of exposures	No. of pigs	No. of serologic responders‡	No. of serologic responders to gD§
VTRS1+ gD	SC	1×10^{10}	3	9	9	7
VTRS1+ gD	Oral	1×10^{10} (x3)	5	10	7	2
RB51+ gD	Oral	1×10^{10} (x3)	5	5	3	3

*Each SC exposure consisted of one dose, while oral vaccinates received three doses per exposure on consecutive days.
**CFU/pig.
‡Western immunoblot using Strain RB51 and VTRS1 cell lysates.
§Western immunoblot using purified gD and Strain RB51+gD cell lysate.

CHAPTER 5: DISCUSSION

Oral vaccination for brucellosis utilizing the rough attenuated vaccine RB51 has been evaluated in several animal species. BALB/c mice and cattle were afforded partial protection against virulent *B. abortus* challenge when orally vaccinated with RB51; however, protection was not as high as that provided by IP or SC RB51 administration (Elzer et al 1998, Stevens et al 1996). Oral RB51 vaccination of dogs was found to be non-pathogenic (Palmer et al 1997), and Lord et al found no differences in the protection provided by oral or IM vaccination of swine to virulent *B. suis* challenge (Lord et al 1998). Vaccination with RB51 has also been evaluated in bison and elk (Cook et al 2002, Olsen et al 2003).

In the current study, oral administration of VTRS1 and RB51 was shown to stimulate the production of antibodies specific to rough *Brucella* antigens in the majority of pigs vaccinated. As expected, the number of serological responders increased with the number of oral exposures to the vaccines, with 100% of RB51 vaccinates sero-positive for rough *Brucella* antigens after the third exposure. The serological response to orally administered VTRS1 was not as high as that of RB51 but also increased over time, with 83% of vaccinates sero-positive for rough *Brucella* antigens ten days following the fourth, and final, exposure. At no time were anti-OPS antibodies detected in any of the vaccinates (Table 1). These data indicate that RB51 and VTRS1 do stimulate host immune responses without interference with diagnostic tests; however, more time may be required to mount a detectable humoral response when swine are orally vaccinated. It is likely that blood samples collected several weeks following the final exposure would yield a higher percentage of VTRS1 vaccinates seropositive for rough *Brucella* antigens.

Previous data collected by Edmonds et al in 2001 showed that SC administration of RB51 resulted in 50% of vaccinates mounting an immune response to rough *Brucella* antigens by day

28 post-vaccination, but colonization of lymph nodes was not detected on days 14 or 28. In contrast, all swine SC vaccinated with VTRS1 in the current study were seropositive for rough *Brucella* antigens by day 24 post-vaccination and the vaccine transiently colonized the swine at low numbers (<200 cfu/g) (Table 2).

A second group of swine were orally vaccinated with VTRS1 utilizing the pecan shell and Karo syrup scarification mechanism developed by Edmonds et al. It was found that despite the use of two logs less CFU than the RB51 vaccination in previous studies (Edmonds et al 2001), VTRS1 stimulated a higher immune response in a shorter period of time. Tissue colonization by RB51 exceeded that of VTRS1; however, VTRS1 again displayed transient colonization similar to that observed in SC vaccinates (Table 2).

Overall, SC and oral administration of VTRS1 resulted in comparable immune responses in vaccinated swine and at no time did the vaccines stimulate the production of anti-OPS antibodies. Lymph node colonization by the vaccine strain was somewhat delayed in oral as compared to SC vaccinates; however, this may be due to the fact that oral exposure requires more time for lymph node colonization localization. Extending the timeframe of this study may offer a better explanation.

Serological testing performed on another set of gilts orally vaccinated with either VTRS1 or RB51 yielded similar immune response results to rough *Brucella* antigens. Eighty-nine percent of VTRS1 vaccinates and 75% of RB51 vaccinates were serological responders following four oral exposures to the vaccines without evidence of the formation of antibodies specific for the OPS antigens of smooth *Brucella* (Table 6). These gilts were bred, challenged at mid-gestation with virulent *B. suis*, and pregnancies were monitored until delivery. All groups, including non-vaccinated saline controls, yielded a comparable average litter size, ranging

between seven and nine piglets. VTRS1 vaccinates and saline control sows had a live birth rate of 94% and 89%, respectively (Table 6). One RB51 vaccinated sow aborted her litter of eleven piglets at approximately 14 weeks gestation. She was not in good health during her pregnancy, apparently due to an abscess on her right hind leg. It is possible that her litter was aborted due to this stress on her pregnancy, as none of the aborted piglets were culture positive for the challenge strain. If her litter is not included in final calculations, 100% of the RB51 vaccinates' litters were born live. It seems that there is no significant difference in live birth rate in vaccinates as compared to saline controls.

In 1995, Hagius et al performed a similar study evaluating the efficacy of orally administered RB51 against virulent *B. suis* challenge. In that study it was found that RB51 vaccination resulted in fewer dead piglets and an increased litter size as compared to saline controls. These results are supported by the present study, in which RB51 vaccinates farrowed an average of eight piglets, as compared to seven piglets in saline controls, and if the aborted litter is not included, 100% of piglets born to RB51 vaccinates were live.

Oral vaccination with both RB51 and VTRS1 were shown to provide approximately three logs of protection against infection of the litter by virulent *B. suis* when the sows were challenged at mid-gestation. The challenge strain was not recovered from any of the RB51 vaccinates' litters, supporting research performed by Hagius et al (1995) and Lord et al (1998). In 2001 the SC administration of VTRS1 was also evaluated in terms of its protection against virulent challenge. None of the vaccinated sows aborted, and both litters and sows were completely protected against virulent *B. suis* challenge (Hagius 2001). Oral vaccination with VTRS1 was shown in the current study to offer less protection than SC vaccination, with 33% of oral VTRS1 vaccinates' litters colonized by the challenge strain at very low numbers (Table 7).

Based on these culture results, it can be stated that the litters of sows orally vaccinated with RB51 or VTRS1 were afforded significant protection against virulent *B. suis* challenge at mid-gestation.

Oral RB51 vaccination was not sufficient to protect sows against infection by virulent *B. suis* when challenged at mid-gestation. All sows were culture positive for the challenge strain four weeks post-parturition (Table 7), in agreement with research performed by Hagius et al in 2001. Lord et al found all RB51 vaccinated sows to be protected against virulent *B. suis* challenge; however, only vaginal swabs were collected for bacteriological analysis (Lord et al 1998). In the current study uterine swabs taken of RB51 vaccinates at necropsy were all negative for *B. suis*, supporting the findings of Lord et al; however, when lymph node colonization was evaluated, 75% of vaccinated sows were positive for *B. suis*. While SC administration of VTRS1 has been shown to provide 100% protection against virulent *B. suis* (Hagius 2001), oral administration in the current study offered partial protection against sow infection. Compared to saline controls, approximately one log of protection was observed in orally vaccinated sows.

Based on these studies, orally administered VTRS1 has proven to be a more efficacious vaccine for swine brucellosis than RB51. Immune responses to rough *Brucella* antigens appear in a shorter period of time after vaccination with VTRS1 as compared to RB51, with transient lymph node tissue colonization. Vaccinated sows were partially protected against virulent *B. suis* challenge at mid-gestation, while RB51 vaccinated sows were not protected. Litters were significantly protected against *B. suis* infection. The absence of anti-OPS antibody formation in VTRS1 vaccinated swine indicates that this vaccine does not interfere with current brucellosis diagnostic tests.

Pseudorabies virus gD was chosen as a candidate for a potential subunit vaccine due to its immunogenic properties. In mice, purified gD has been shown to stimulate the production of virus-neutralizing antibodies (Ishii et al 1988) and protect against PRV infection (Haagmans et al 1999). Marchioli et al was able to administer gD via a recombinant vaccinia virus and provide protection against lethal PRV infection in both mice and swine (Marchioli et al 1987), and Shiau et al used live non-pathogenic *E. coli* as a delivery method to induce protective immune responses against gD in mice (Shiau et al 2001). In order to create a multivalent vaccine for PRV and brucellosis, the plasmid pBBgroE ssLgD, encoding for gD, was successfully electroporated into RB51 and VTRS1 in the current study (Figure 1), yielding vaccine strains RB51+gD and VTRS1+gD. These multivalent vaccines required an additional 48 hours for appearance of colonies in the laboratory as compared to the parent strains.

Serological responses in swine SC vaccinated with RB51+gD were greater than RB51 vaccinates in previous studies performed by Edmonds et al in 2001. One hundred percent of RB51+gD vaccinated swine were seropositive for rough *Brucella* antigens by day 24 (Table 3), whereas only 50% of SC RB51 vaccinates were positive. At no point were antibodies specific for the OPS antigens of smooth *Brucella* detected in any of the vaccinates. Lymph node colonization by the vaccine strains was comparable in both RB51 and RB51+gD vaccinates, yielding low numbers of the vaccine strains (Edmonds et al 2001).

Oral vaccination with RB51+gD yielded serological responses comparable to that stimulated by oral administration of the parent strain, RB51, showing that the addition of the pBBgroE ssLgD plasmid neither alters the stimulation of antibodies to rough *Brucella* antigens, nor provokes immune responses to OPS antigens. Approximately two logs greater CFU was used for the RB51 oral vaccination performed by Edmonds et al in 2001, which most likely

explains the lower levels of tissue colonization present in RB51+gD vaccinated swine. It is also possible that the slower growth *in vitro* of the transformed strain decreased the number of colonized swine in this study. Extending the timeframe of the study in order to observe tissue colonization over a longer period of time post-vaccination may allow for a clearer interpretation of this data.

Both SC and oral vaccination with VTRS1+gD provided comparable stimulation of the formation of antibodies against rough *Brucella* antigens as the parent strain VTRS1 without stimulating the production of anti-OPS antibodies; however, VTRS1+gD was not recovered from the lymph nodes of vaccinated swine until at least 24 days post-vaccination when administered either orally or SC (Table 4). This delayed colonization may also be due to slower growth of the transformed bacteria, and evaluation of lymph nodes beyond 31 days post vaccination may allow for further clarification.

Previous studies have shown that gD stimulates humoral immune responses and the formation of virus-neutralizing antibodies (Haagmans et al 1999, Ishii et al 1988), and when administered via live vaccinia virus or *E. coli*, gD partially protected mice against virulent PRV challenge (Marchioli et al 1987, Shiau et al 2001). In the current study, SC VTRS1+gD vaccination provided the highest immune responses to PRV gD, with 66% of vaccinates sero-positive for antibodies against PRV gD (Table 4). It is surprising that greater immune responses were not detected in the current study; only 33% of SC RB51+gD vaccinates and 14% of oral RB51+gD and VTRS1+gD vaccinates were sero-positive for antibodies against PRV gD (Tables 3 and 4). The low number of vaccinates forming antibodies to the plasmid protein may be linked to the slower growth of the transformed vaccine strains.

When administered to pregnant swine at mid-gestation, neither VTRS1+gD nor RB51+gD were abortogenic, and the vaccine strains were not recovered from the sows or their litters. Additionally, challenge with the transformed strains did not decrease litter size or the live birth rate, as compared to saline controls (Table 5). Two logs greater CFU were used to challenge sows with the vaccine strains due to their rough morphology and suspected attenuated nature, and even with this increased dose, VTRS1+gD and RB51+gD were shown to be safely administered to pregnant sows.

The efficacy of these vaccines has not yet been fully evaluated, as the final study is ongoing; however, the serological responses of gilts vaccinated prior to breeding have been examined. One hundred percent of gilts SC vaccinated with VTRS1+gD were seropositive for rough *Brucella* antigens after two exposures without stimulating the production of antibodies specific for the OPS antigens of smooth *Brucella*, and 78% possessed antibodies to the PRV gD. The orally administered strains yielded lower immune responses than their parent strains, even with a total of five exposures to the vaccines (Table 8). This is likely attributed to their slower growth in the host as noted both in the laboratory and when lymph node colonization was evaluated.

A concise evaluation of the use of RB51+gD and/or VTRS1+gD as vaccines against brucellosis cannot be given until the efficacy study is complete; however, it appears that while lymph node colonization is not as efficient as with the parent strains, serological responses to rough *Brucella* antigens are not altered by the addition of the pBBgroE ssLgD plasmid, these vaccines do not interfere with current brucellosis diagnostic tests by stimulating the formation of anti-OPS antibodies, and both strains appear to be safe for use in pregnant swine.

CHAPTER 6: SUMMARY

In this study it has been found that the rough attenuated mutant *B. suis* Strain VTRS1 stimulates host immune responses when administered either orally or SC to swine as well as transiently colonizing lymph node tissues without interfering with current brucellosis diagnostic techniques. The antibody production specific for rough *Brucella* antigens stimulated by VTRS1 vaccination surpasses that present in rough *B. abortus* Strain RB51 vaccinates. Additionally, VTRS1 provides significant protection against litter infection when orally vaccinated sows are challenged with virulent *B. suis* at mid-gestation. Both RB51 and VTRS1 vaccination provide approximately three logs of protection against litter infection; however, unlike RB51, VTRS1 also provides the sows themselves with partial protection against virulent *B. suis* challenge. These data support the conclusion that VTRS1 is a more efficacious vaccine than RB51 for swine brucellosis and does not interfere with current brucellosis diagnostic techniques.

The addition of a plasmid encoding for PRV gD to both RB51 and VTRS1 has no significant effect on the ability of these strains to stimulate humoral immune responses to rough *Brucella* antigens in vaccinated swine and does not interfere with brucellosis diagnostic techniques; however, despite the highly immunogenic nature of gD, low numbers of swine produce detectable immune responses to this antigen as a result of oral or SC RB51+gD or VTRS1+gD vaccination. Additionally, tissue colonization in RB51+gD and VTRS1+gD vaccinated swine is significantly lower than that present in pigs vaccinated with the parent strains. These observations can most likely be attributed to the slower growth of RB51+gD and VTRS1+gD in the host. Future studies focusing on tissue colonization over longer periods of time post-vaccination are necessary in order to determine the cause of this phenomenon.

VTRS1+gD and RB51+gD do not cause abortion or infection of the sow or litter when administered conjunctively to pregnant sows at mid-gestation, indicating that these vaccines are safe for use in pregnant swine. Experiments evaluating the efficacy of RB51+gD and VTRS1+gD against *B. suis* challenge are currently in progress. Based on the fact that no decrease in immune response against rough *Brucella* antigens is observed following the addition of the gD plasmid, sow and litter protection against challenge with virulent *B. suis* in RB51+gD and VTRS1+gD vaccinated sows is expected to be equivalent to that afforded by the parent strains.

Future studies are necessary in order to completely evaluate RB51+gD and VTRS1+gD as multivalent vaccines for brucellosis and pseudorabies in swine. These vaccines must be evaluated in terms of their efficacy against virulent PRV challenge in both sows and their litters, as well as finding means to increase host immune responses to the gD antigen.

VTRS1 has proven to surpass RB51 in its ability to provoke vaccinates' immune responses without interfering with diagnostic tests for brucellosis as well as provide greater protection against sow infection following mid-gestational challenge with virulent *B. suis*. The addition of a plasmid encoding for PRV gD to RB51 and VTRS1 does not affect host immune responses to rough *Brucella* antigens, though the formation of anti-PRV gD antibodies stimulated by vaccination with RB51+gD and VTRS1+gD is lower than expected.

The feral swine reservoirs that harbor *B. suis* and PRV remain a constant threat to domestic herds. A multivalent vaccine that could be administered orally to the feral swine population as bait would greatly increase the success of brucellosis and pseudorabies eradication programs, as well as protect domestic herds from infection. The efficacy of the vaccine strain *B. suis* Strain VTRS1 and its lack of interference with current brucellosis diagnostic tests make it an

excellent candidate for a swine brucellosis vaccine. The multivalent vaccine strains RB51+gD and VTRS1+gD stimulate host immune responses to rough *Brucella* antigens and are safe for use in pregnant swine. These strains are expected to demonstrate the same level of protection against virulent *B. suis* challenge observed in parent strains RB51 and VTRS1; however immune responses to PRV gD in vaccinated swine and the efficacy of RB51+gD and VTRS1+gD against virulent PRV challenge in pregnant swine must further be evaluated prior to their use as multivalent vaccines.

REFERENCES

Aiello, S. E. (ed.). 1998. Pseudorabies, p. 964-966. *In* The Merck Veterinary Manual, 8th Ed. Merck and Co., Inc., Whitehouse Station, NJ.

Alton, G. G. 1990. *Brucella suis*, p. 411-421. *In* K. Nielsen and J. R. Duncan (ed.), Animal Brucellosis. CRC Press, Inc., Boca Raton, FL.

Alton, G. G., L. A. Corner, and P. Plackett. 1980. Vaccination of Pregnant Cows with Low Doses of *Brucella abortus* Strain 19 Vaccine. Aust. Vet. J. 56:369-372.

Beckett, F. W., and S. C. MacDiarmid. 1987. Persistent Serological Titres Following Reduced Dose *Brucella abortus* Strain 19 Vaccination. Br. Vet. J. 143:477-478.

Bosseray, N., and M. Plommet. 1990. *Brucella suis* S2, *Brucella melitensis* Rev. 1, and *Brucella abortus* S19 Living Vaccines: Residual Virulence and Immunity Induced Against Three *Brucella* Species Challenge Strains in Mice. Vaccine. 8:462-468.

Bundle, D. R., J. W. Cherwonogrodzky, M. Caroff, and M. B. Perry. 1987. The Lipopolysaccharides of *Brucella abortus* and *B. melitensis*. Ann Inst. Pasteiu/Microbiol. 138:92-98.

Cherwonogrodzky, J. W., G. Dubray, E. Moreno, and H. Mayer. 1990. Antigens of *Brucella*, p. 19-56. *In* K. Nielsen and J. R. Duncan (ed.), Animal Brucellosis. CRC Press, Inc., Boca Raton, FL.

Cheville, N. F., A. E. Jensen, S. M. Halling, F. M. Tatum, D. C. Morfitt, R. G. Hennager, W. M. Frerichs, and G. Schurig. 1992. Bacterial Survival, Lymph Node Changes, and Immunologic Responses of Cattle Vaccinated with Standard and Mutant Strains of *Brucella abortus*. Am. J. Vet. Res. 53:1881-1888.

Cheville, N. F., S. C. Olsen, A. E. Jensen, M. G. Stevens, M. V. Palmer, and A. M. Florance. 1996. Effects of Age at Vaccination on Efficacy of *Brucella abortus* Strain RB51 to Protect Cattle Against Brucellosis. Am. J. Vet. Res. 57:1153-1156.

Confer, A. W., S. M. Hall, C. B. Faulkner, B. H. Espe, B. L. Deyoe, R. J. Morton, and R. A. Smith. 1985. Effects of Challenge Dose on the Clinical and Immune Responses of Cattle Vaccinated with Reduced Doses of *Brucella abortus* Strain 19. Vet. Microbiol. 10:561-575.

Conger, T. H., E. Young, and R. A. Heckmann. 1999. *Brucella suis* in Feral Swine, p. 98-107. Proc. of the Feral Swine Symposium. Texas Animal Health Commission, Ft. Worth, TX.

Cook, W. E., E. S. Williams, E. T. Thorne, T. J. Kreeger, G. Stout, K. Bardsley, H. Edwards, G. Schurig, L. A. Colby, F. Enright, and P. H. Elzer. 2002. *Brucella abortus* Strain RB51 Vaccination in Elk. I. Efficacy of Reduced Dosage. J. Wildl. Dis. 38:18-26.

Dees, T. 1999. Feral/Wild Swine Surveillance for Foreign Animal Diseases and Some Field Study Projects on Brucellosis and Pseudorabies in the Southeastern USA, p. 121-123. Proc. of the Feral Swine Symposium. Texas Animal Health Commission, Ft. Worth, TX.

DelVecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernai, M. Mazur, E. Goltsman, E. Selkov, P. H. Elzer, S. Hagius, D. O'Callaghan, J. Letesson, R. Hasselkorn, N. Kyrpides, and R. Overbeek. 2002. The Genome Sequence of the Facultative Intracellular Pathogen *Brucella melitensis*. Proc. Nat. Acad. Sci. 99:443-448.

Deyoe, B. L., and C. A. Manthei. 1975. Brucellosis, p. 492-515. *In* H. W. Dunne and A. D. Leman (ed.), Diseases of Swine, 4th Ed. The Iowa State University Press, Ames, Iowa.

Edmonds, M. D., L. E. Samartino, P. G. Hoyt, S. D. Hagius, J. V. Walker, F. M. Enright, G. G. Schurig, and P. Elzer. 2001. Oral Vaccination of Sexually Mature Pigs With *Brucella abortus* Vaccine Strain RB51. Am. J. Vet. Res. 62:1328-1331.

Eliot, M., D. Fargeaud, R. L'Haridon, and B. Toma. 1988. Identification of the Pseudorabies Virus Glycoprotein gp50 as a Major Target of Neutralizing Antibodies. Arch. Virol. 99: 45-56.

Elzer, P. H., F. M. Enright, L. Colby, S. D. Hagius, J. V. Walker, M. B. Fatemi, J. D. Kopec, V. C. Beal, and G. G. Schurig. 1998. Protection Against Infection and Abortion Induced by Virulent Challenge Exposure After Oral Vaccination of Cattle with *Brucella abortus* Strain RB51. Am. J. Vet. Res. 59:1575-1578.

Elzer, P. H., M. E. Kovach, R. W. Phillips, G. T. Robertson, K. M. Peterson, and R. M. Roop II. 1995. *In Vivo* and *in Vitro* Stability of the Broad-Host-Range Cloning Vector pBBR1MCS in Six *Brucella* Species. Plasmid. 33:51-57.

Enright, F. M. 1990. The Pathogenesis and Pathobiology of *Brucella* Infection in Domestic Animals, p. 301-318. *In* K. Nielsen and J. R. Duncan (ed.), Animal Brucellosis. CRC Press, Inc., Boca Raton, FL.

Goode, E. R., Jr., C. A. Manthei, G. E. Blake, and T. E. Amerault. 1952. *Brucella suis* Infection in Suckling and Weanling Pigs. II. J. Am. Vet. Med. Assoc. 121:456-464.

Graves, R. R. 1943. The Story of John M. Buck's and Matilda's Contribution to the Cattle Industry. J. Am. Vet. Med. Assoc. 102:193-195.

Gustafson, D. P. 1981. Pseudorabies, p. 209-223. *In* A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), Diseases of Swine, 5th Ed. Iowa State University Press, Ames, Iowa.

Haagmans, B. L., E. M. A. van Rooij, M. Dubelaar, T. G. Kimman, M. C. Horzinek V. E. C. J. Schijns, and A. T. J. Bianchi. 1999. Vaccination of Pigs Against Pseudorabies Virus with Plasmid DNA Encoding Glycoprotein D. Vaccine. 17:1264-1271.

Hagius, S. D., J. V. Walker, M. B. Fatemi, P. G. Hoyt, G. G. Schurig, L. Colby, J. F. Annelli, F. M. Enright, and P. H. Elzer. 1995. Evaluation of *Brucella abortus* Strain RB51 as an Oral Vaccine Candidate in Swine, abstr. 139. *In* Abstracts of the 76th Annual Meeting of the Conference of Research Workers in Animal Diseases. Iowa State University Press, Ames, Iowa.

Hagius, S., T. Fulton, F. Enright, J. Walker, P. Hoyt, G. Schurig, and P. Elzer. 2001. A Promising Vaccine for Swine Brucellosis, abstr. P13. *In* Abstracts of the 54th Annual Brucellosis Research Conference. St. Louis, Missouri.

Hahn, E. C., G. R. Page, P. S. Hahn, K. D. Gillis, C. Romero, J. A. Annelli, and E. P. J. Gibbs. 1997. Mechanisms of Transmission of Aujeszky's Disease Virus Originating from Feral Swine in the USA. Vet. Microbiol. 55:123-130.

Hanssens, F. P., H. J. Nauwynck, and T. C. Mettenleiter. 1995. Role of Glycoprotein gD in the Adhesion of Pseudorabies Virus Infected Cells and Subsequent Cell-Associated Virus Spread. Arch. Virol. 140: 1855-1862.

Hanson, R. P. 1954. The History of Pseudorabies in the United States. J. Am. Vet. Med. Assoc. 124:259-261.

Horn, F. P., and R. G. Breeze. 1999. Agriculture and Food Security. Ann. N. Y. Acad. Sci. 894:9-17.

Hurvell, B. 1973. Serological Cross-Reactions Between Different *Brucella* Species and *Yersinia enterocolitica.* Biological and Chemical Investigations of Lipopolysaccharides from *Brucella abortus* and *Yersinia enterocolitica* type IX. Acta. Pathol. Microbiol. Scand. Sect. B. 81:113-119.

Iglesias, J. G. 1993. Pseudorabies Virus Infection in Swine: Unsuspected Ways of Transmission and Perpetuation, p. 185-190. *In* Infectious Disease in Food Animal Practice. Veterinary Learning Systems, Trenton, NJ.

Ishii, H., Y. Kobayashi, M. Kuroki, and Y. Kodama. 1988. Protection of Mice from Lethal Infection with Aujeszky's Disease Virus by Immunization with Purified gVI. J. Gen. Virol. 69:1411-1414.

Jimenez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter. 1994. Vaccination with *Brucella abortus* Rough Mutant RB51 Protects BALB/c Mice Against Virulent Strains of *Brucella abortus, Brucella melitensis,* and *Brucella ovis*. Infec. Immun. 62:4990-4996.

Kortepeter, M. G., and G. W. Parker. 1999. Potential Biological Weapons Threats. Emerg. Infect. Dis. 5:523-527.

Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson. 1994. pBBR1MCS: A Broad-Host-Range Cloning Vector. BioTechniques. 16:800.

Kreutzer, D. L., and D. C. Robertson. 1979. Surface Macromolecules and Virulence in Intracellular Parasitism: Comparison of Cell Envelope Components of Smooth and Rough Strains of *Brucella abortus*. Infec. Immun. 23:819-828.

Lai, F., G. G. Schurig, and S. M. Boyle. 1990. Electroporation of a Suicide Plasmid Bearing a Transposon into *Brucella abortus*. Microb. Pathogen. 9:363-368.

Lawhorn, B. 1988. A New Approach for Obtaining Blood Samples from Pigs. J. Am. Vet. Med. Assoc. 192:781-782.

Lawson, J. R. 1950. Strain 19 and the Control of Brucellosis. Vet. Rec. 62:823-830.

Lee, J. Y. S., and M. R. Wilson. 1979. A Review of Pseudorabies (Aujeszky's Disease) in Pigs. Can. Vet. J. 20:65-69.

Leman, A. D., M. Cropper, and H. E. Rodeffer. 1974. Infectious Swine Reproductive Diseases. Theriogenology. 2:149-160.

Lindley, D. C., and J. R. Lander. 1949. Observations on the Use of *Brucella abortus* Strain 19 in Swine – Including Multiple Intradermal Doses. J. Am. Vet. Med. Assoc. 115:359-363.

Lord, V. R., J. W. Cherwonogrodzky, G. G. Schurig, R. D. Lord, M. J. Marano, and G. E. Melendez. 1998. Venezuelan Field Trials of Vaccines Against Brucellosis in Swine. Am. J. Vet. Res. 59:546-551.

MacMillan, A. 1990. Conventional Serological Tests, p. 153-197. *In* K. Nielsen and J. R. Duncan (ed.), Animal Brucellosis. CRC Press, Inc., Boca Raton, FL.

Manthei, C. A. 1968. Brucellosis as a Cause of Abortion Today, p. 8-94. *In* L. C. Faulkner (ed.), Abortion Diseases of Livestock. Charles C. Thomas, Springfield, IL.

Manthei, C. A., C. K. Mingle, and R. W. Carter. 1952. *Brucella suis* Infection in Suckling and Weanling Pigs. I. J. Am. Vet. Med. Assoc. 121:373-382.

Marchioli, C. C., R. J. Yancey, Jr., E. A. Petrovskis, J. G. Timmins, and L. E. Post. 1987. Evaluation of Pseudorabies Virus Glycoprotein gp50 as a Vaccine for Aujeszky's Disease in Mice and Swine: Expression by Vaccinia Virus and Chinese Hamster Ovary Cells. J. Virol. 61:3977-3982.

McDiarmid, A. 1957. The Degree and Duration of Immunity in Cattle Resulting from Vaccination with S. 19 *Br. abortus* Vaccine and its Implication in the Future Control and Eventual Eradication of Brucellosis. Vet. Rec. 69:877-879.

Mettenleiter, T. C. 1991. Molecular Biology of Pseudorabies (Aujeszky's Disease) Virus. Comp. Immun. Microbiol. Infect. Dis. 14: 151-163.

Mettenleiter, T. C. 1996. Immunobiology of Pseudorabies (Aujeszky's Disease). Vet. Immunol. Immunopathol. 54:221-229.

Meyer, M. E. 1990. Current Concepts in the Taxonomy of the Genus *Brucella*, p. 1-13. *In* K. Nielsen and J. R. Duncan (ed.), Animal Brucellosis. CRC Press, Inc., Boca Raton, FL.

Mulder, W. A. M., J. M. A. Pol, E. Gruys, L. Jacobs, M. C. M. De Jong, B. P. H. Peeters, and T. G. Kimman. 1997. Pseudorabies Virus Infections in Pigs. Role of Viral Proteins in Virulence, Pathogenesis, and Transmission. Vet. Res. 28: 1-17.

Nicoletti, P. 1984. Vaccination of Cattle with *Brucella abortus* Strain 19 Administered by Differing Routes and Doses. Vaccine. 2:133-135.

Nicoletti, P. 1990. Vaccination Against *Brucella*. Adv. Biotechnol. Processes. 13:147-168.

Olsen, S. C., A. E. Jensen, W. C. Stoffregen, and M. V. Palmer. 2003. Efficacy of Calfhood Vaccination with *Brucella abortus* Strain RB51 in Protecting Bison Against Brucellosis. Res. Vet. Sci. 74:17-22.

Palmer, M. V., and N. F. Cheville. 1997. Effects of Oral or Intravenous Inoculation with *Brucella abortus* Strain RB51 Vaccine in Beagles. Am. J. Vet. Res. 58:851-856.

Palmer, M. V., S. C. Olsen, and N. F. Cheville. 1997. Safety and Immunogenicity of *Brucella abortus* Strain RB51 Vaccine in Pregnant Cattle. Am. J. Vet. Res. 58:472-477.

Paulsen, I. T., R. Seshadri, K. E. Nelson, J. A. Eisen, J. F. Heidelberg, T. D. Read, R. J. Dodson, L. Umayam, L. M. Brinkac, M. J. Beanan, S. C. Daugherty, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, W. C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. E. Van Aken, S. Riedmuller, H. Tettelin, S. R. Gill, O. White, S. L. Salzberg, D. L. Hoover, L. E. Lindler, S. M. Halling, S. M. Boyle, and C. M. Fraser. 2002. The *Brucella suis* Genome Reveals Fundamental Similarities Between Animal and Plant Pathogens and Symbionts. Proc. Nat. Acad. Sci. 99:13148-13153.

Romero, C. H., P. N. Meade, J. E. Shultz, H. Y. Chung, E. P. Gibbs, E. C. Hahn, and G. Lollis. 2001. Venereal Transmission of Pseudorabies Viruses Indigenous to Feral Swine. J. Wild. Dis. 37:289-296.

Schurig, G. G., N. Sriranganathan, and M. J. Corbel. 2002. Brucellosis Vaccines: past, present, and future. Vet. Microbiol. 90:479-496.

Schurig, G. G., R. M. Roop, II, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan. 1991. Biological Properties of RB51; a stable rough strain of *Brucella abortus*. Vet. Microbiol. 28:171-188.

Shiau, A., C. Chu, W. Su, and C. Wu. 2001. Vaccination with the Glycoprotein D gene of Pseudorabies Virus Delivered by Nonpathogenic *Escherichia coli* Elicits Protective Immune Responses. Vaccine. 19:3277-3284.

Stevens, M. G., S. C. Olsen, M. V. Palmer, and G. W. Pugh. 1996. Immune Responses and Resistance to Brucellosis in Mice Vaccinated Orally with *Brucella abortus* RB51. Infec. Immun. 64:4534-4541.

Timony, J. F., J. H. Gillespie, F. W. Scott, and J. E. Barlough. 1988. The Genus *Brucella*, p. 135-152. *In* Hagan and Bruner's Microbiology and Infectious Diseases of Domestic Animals, 8th ed. Comstock Publishing Associates, Ithaca, NY.

Tobias, L., G. G. Schurig, and D. O. Cordes. 1992. Comparative Behaviour of *Brucella abortus* Strains 19 and RB51 in the Pregnant Mouse. Res. Vet. Sci. 53:179-183.

Winter, A. J., G. G. Schurig, S. M. Boyle, N. Sriranganathan, J. S. Bevins, F. M. Enright, P. H. Elzer, and J. D. Kopec. 1996. Protection of BALB/c Mice Against Homologous and Heterologous Species of *Brucella* by Rough Strain Vaccines Derived from *Brucella melitensis* and *Brucella suis* biovar 4. Am. J. Vet. Res. 57:677-683.

Xin, X. 1986. Orally Administrable Brucellosis Vaccine: *Brucella suis* Strain 2 Vaccine. Vaccine. 4:212-216.

VITA

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